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Army Medical College Epidemiological
Research Report

Section 2 Number 324

Relationship of Supersonic Wave Frequency and Cellulicidal Action

Part 1. Supersonic Wave Generator; Experimentation with Cholera Bacteria

Army Medical College Epidemiology Laboratory

(Maj. Gen. ISHII, commanding)

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Table of Contents

General

Numerous studies have been performed on the effects of supersonic waves against different types of bacteria. In the study of cholera bacteria alone (1) WATANABE and his fellow researchers discovered that antigens produced by destroying cholera bacteria with supersonic waves were in comparison to ordinary bacterial solutions weaker in toxicity, and that their agglutinin-complement fixation properties, antibodies and immunifacient powers contributed favorably toward producing immunity; (2) OKITSU observed in his complement fixation reaction tests that a bacterial solution treated for 20 to 30 minutes with supersonic waves was most satisfactory and produced results superior to those of past antigens; and (3) OZAKI reported that the cellulicidal action of supersonic waves is governed to a considerable degree by temperature, that temperature rises destroy bacteria, but produce a decrease in transparency and that turbidity is not pronounced at 30°C or below.

Each of the above studies was performed on a single frequency. The effects produced by varying the frequency have not been investigated. The purpose of this experiment was to make comparative studies on the effect of different frequencies (1120 kc, 560 kc and 280 kc) on bacterial cells and to clarify the relationship between frequency and cellulicidal action.

Chapter I. Supersonic wave generator; application of supersonic waves.

A. Supersonic wave generator: The supersonic wave generator (Model Number 2) employed by this laboratory for this experiment was designed on October 1938 upon the request of the late Dr. WATANABE, Watari. Construction was completed last year. (Army Medical College Epidemiology Laboratory Report Number II-36.) The principal features of the generator are described below.

1. The range covers five frequencies (140 kc, 280 kc, 560 kc, 1120 kc and 2240 kc).

2. Two resonators have been provided; both can be oscillated simultaneously.

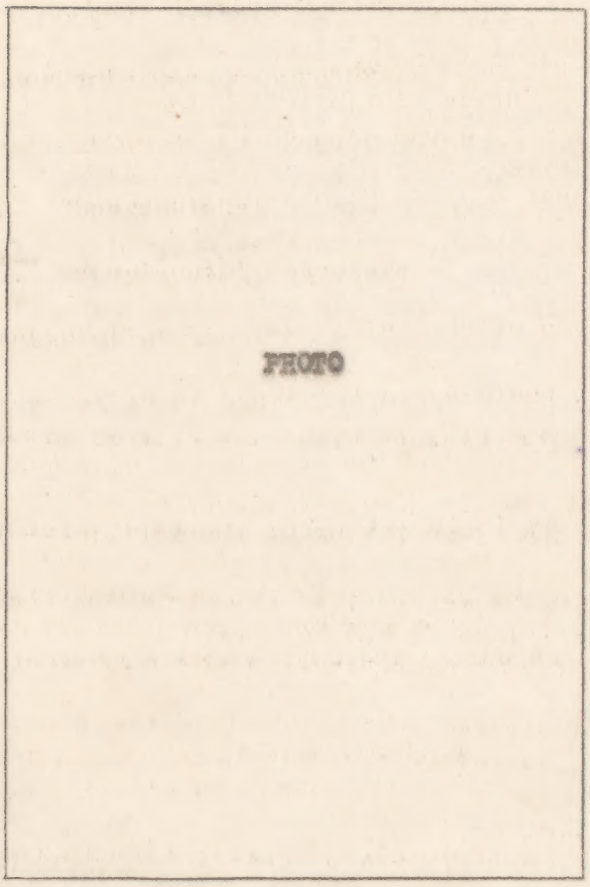
3. Increased dielectric strength around the crystal resonators is obtained by the automatic temperature control and the oil purifier which is provided for the insulating oil.

4. The plate input of the oscillator tube (one per crystal resonator) is fixed at 1.5 times or 1.5 kw.

5. Compensators allow for voltage fluctuations in the power supply.

Supersonic wave generator

Front view (Part 1)



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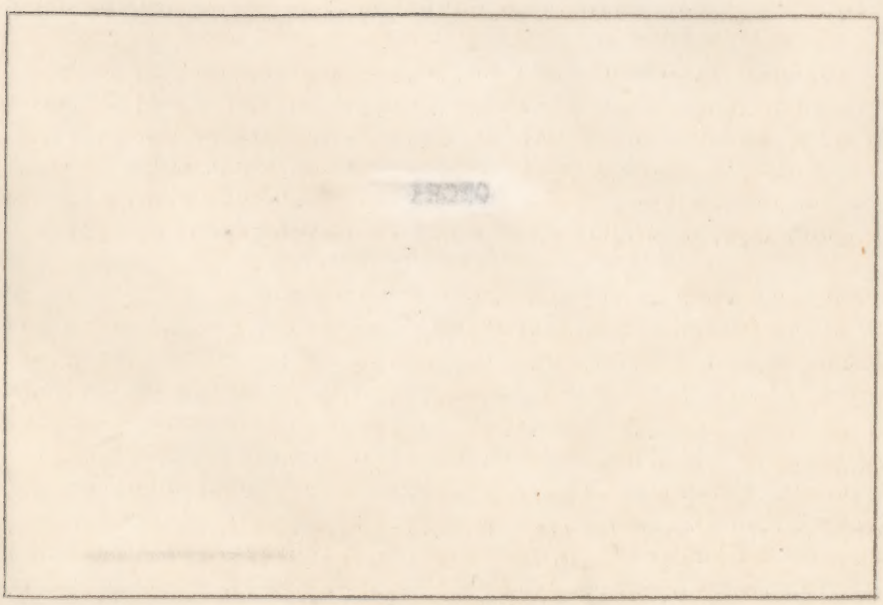
Supersonic wave generator

Front view (Part 2)



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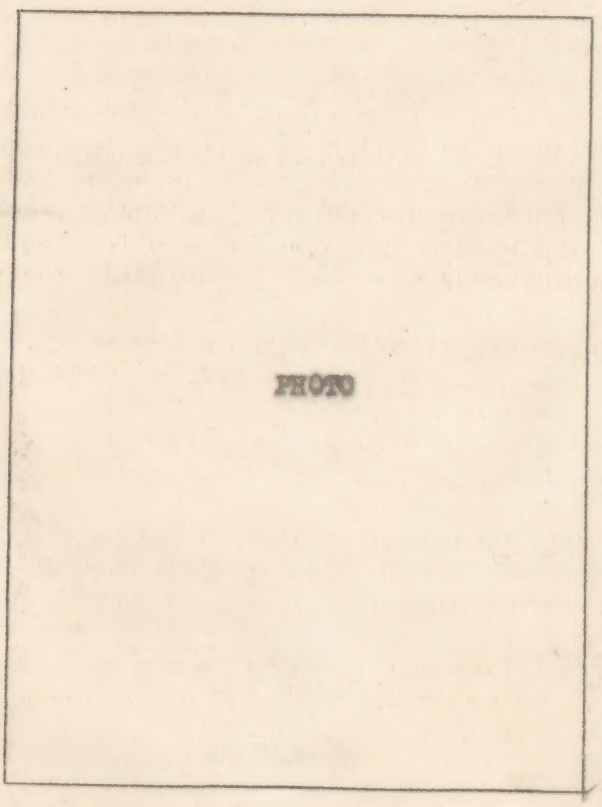
4½"

The bacterial solution containers were redesigned by this laboratory. Ordinarily it has been the practice in supersonic wave treatments to place the bacterial solution in a thin-bottomed test tube which in turn was immersed in the oil froth formed on the oil surface above the crystal resonator. Under such conditions the bacterial solution is sprayed upward by the supersonic waves. Because the spray droplets adhere to the test-tube wall a considerable length of time elapses before these droplets descend to the surface of the bacterial solution. Though some of the force from the supersonic waves passes through the test-tube wall and acts on the clinging droplets the intensity is considerably less than that acting on the solution lying at the bottom. When the supersonic wave treatment is discontinued after a fixed period the portion of the bacterial solution which received a smaller amount of the treatment mixes with the portion at the bottom. This leads to inaccuracies in determining the time required to destroy bacteria.

A special container built according to specifications described below was used in the experiment in order to reduce such inaccuracies.

Special container employed
in experiment



Hard glass was used in making the special container. Based on past experience a 0.5 mm thickness at the bottom section was determined to be sufficient. The bacterial solution was placed in part A at the lower end of the small inside tube, the length of which was set according to the volume of bacterial solution to be handled.

An opening approximately 20 mm in diameter to be used in injecting or extracting the bacterial solution was provided on the hemispherical surface at the top of this tube. This enables the majority of the spray droplets to deflect downward from the opening toward the tube wall and drop rapidly. The droplets which pass through the opening and strike the wall of the larger tube descend to part B and cannot mix with the solution at the bottom.

This container was tested at our laboratory last spring. The tests revealed a slight shortening of the time required to destroy bacteria.

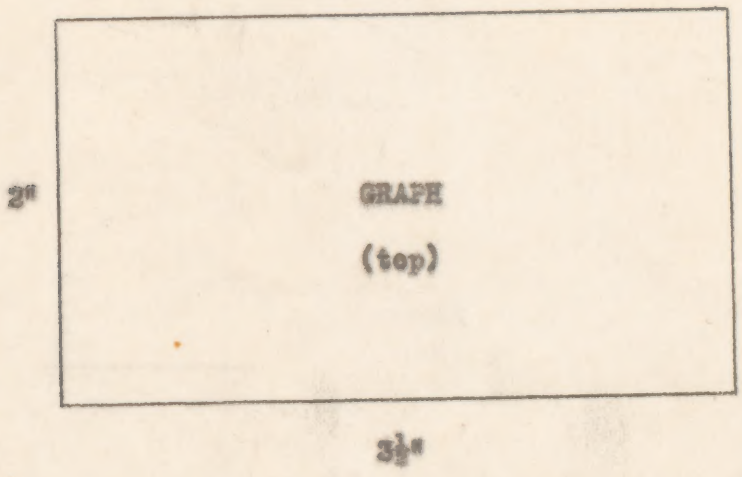
B. Application of supersonic waves: As stated previously comparative studies on the effects of supersonic waves on bacterial solutions were performed at three frequencies (1120 kc, 560 kc and 280 kc). In order to achieve a constant supersonic wave output 50 cc of transformer oil was placed in the above-mentioned special container and the oscillator was tuned to produce at each frequency a 10°C per minute rise in oil temperature. (Figure 2 illustrates an example produced when tuning was accomplished in such a manner.) Other experimental conditions are listed below.

Frequency	Wave length	Plate voltage	Plate current	Grid current
(kc)	(m)	(v)	(ma)	(ma)
1120	268	3000	460	165
560	535	3000	550	130
280	1070	3300	400	190

Crystal resonator position:	5 cm below oil surface
Bacterial solution container position:	Container bottom 3 cm above oil surface
Oil bath temperature (internal):	30°C
Bacterial solution temperature (constant):	42°C (± 2°C)
Bacterial solution volume:	100 cc

Under conditions where the initial liquid (oil) temperature closely approaches the ambient temperature, the result shown in Figure 1 is obtained by plotting the treatment time and the temperature of the transformer oil contained in the special test tube when supersonic waves act against the bottom of the tube.

Figure 1



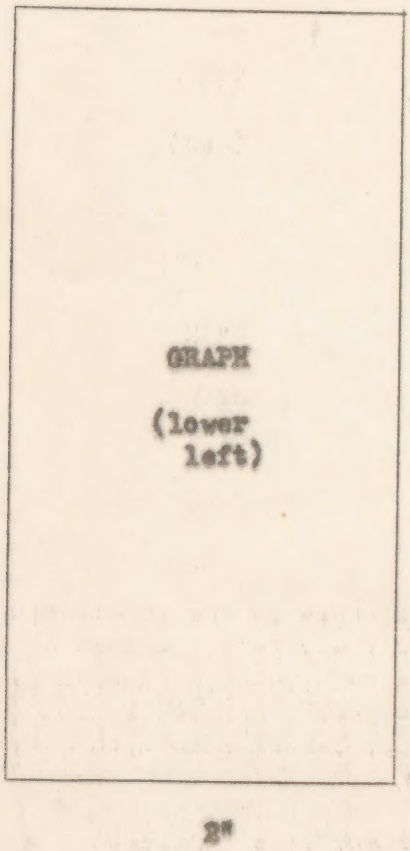
Key

(1) Test tube temperature (internal)

(2) Treatment time (minutes)

The straight temperature rise during the initial stage indicates that the heat generated through the absorption of supersonic waves is considerably greater than the thermal conductivity within and without the tube. The rate of temperature rise in this portion is proportionate to the supersonic wave input inside of the tube.

Figure 2. Bacterial count variations of 1 mg/l cc bacterial (cholera) solution



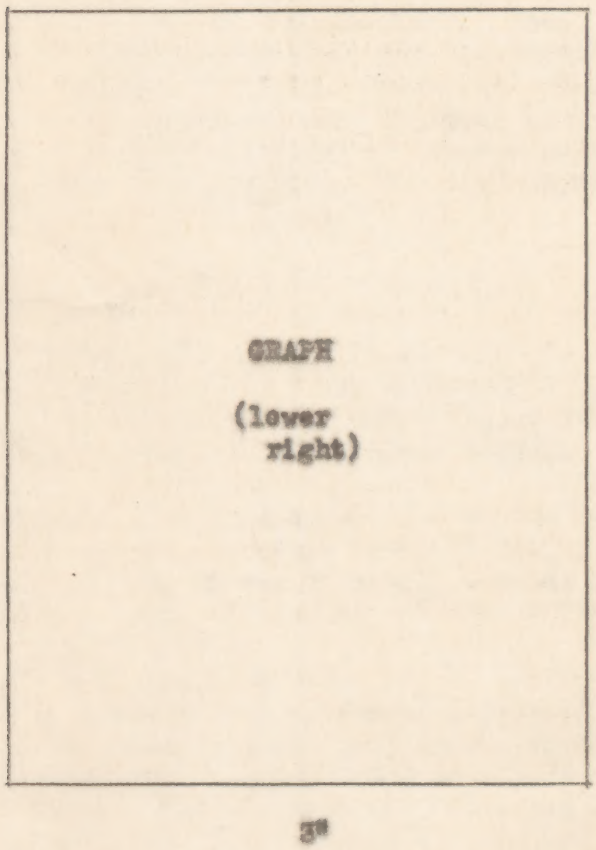
Key

(1) Bacterial count

(2) Stock solution

(3) Time (minutes)

Figure 3. Bacterial count variations of 10 mg/1 cc bacterial (cholera) solution



Key

(1) Bacterial count

(2) Stock solution

(3) Time (minutes)

Chapter II. Observations

The items listed below were observed on bacterial suspensions containing 0.1 mg, 1.0 mg and 10 mg of cholera bacteria per 1.0 cc. These observations were made at different frequencies.

A. Cellulicidal test

B. Bacterial count

C. Turbidity measurement

D. Time change in morphology

Chapter III. Experimental procedure

A. Bacterial strain: The bacteria used in this experiment were of the Kitani strain preserved by this laboratory. The strain was always subjected to animal passage before use. Virulence against mice was 0.1 mg. Uniform turbidity and "bacterial film" resulted with a bouillon culture (PH 7.4). "Bacterial film," uniform turbidity and excellent growth were observed in peptone. Gas formation with a vertical dextrose or lactose culture was negative. Cholera-red reaction was positive. The agglutination titer of the original serum was positive at 12,800 times.

B. Preparation of bacterial suspensions: The product separating after 24 hours in 3 per cent agar (PH 7.4) was cultured at 37°C for 20 hours and weighed. Bacterial suspensions were prepared at a ratio of 10 mg per cc of 0.85 per cent physiological saline solution. The suspensions were employed after completing tests for miscellaneous bacteria and bacterial counts.

C. Supersonic wave treatment: The 0.1 mg bacterial suspension was treated with supersonic waves for 1-10 minute periods, the 1.0 mg suspension for 1-10, 15 and 20 minute periods and the 10.0 mg suspension for 1-10, 20, 30, 40, 50, 60 and 70 minute periods.

D. Qualitative tests on cellulicidal strength: Immediately following the supersonic wave treatment bacterial suspensions from the respective treatment periods were slant-cultured in 3 per cent agar and plate-cultured in peptone and agar media with large platinum-wire loops. The cultures were observed after 20 hours (48 hours if growth was insufficient) at 37°C and their development was graded with symbols ++, +, + and −.

E. Bacterial count: The supersonic wave-treated bacterial solutions were diluted progressively ten times each. One cc of each dilution was placed in a Petri dish into which 10 cc of 45°C agar was later added. Upon solidification, agar was again added in a proportion sufficient to cover the surface with a thin film. Each was examined after a 24 hour, 37°C culture; colony counts were made after a 48-hour period if growth was inadequate at that time.

Counts on the number of destroyed bacteria were taken on the control and also during the intermediate stage and the stage prior to destruction. The rate of time decrease compared to the control as well as their logarithmic values were obtained following the bacterial count. These included the number of developed colonies per milligram, the percentage of developed colonies in comparison to the control and the logarithmic values of the bacterial count.

F. Observation of morphological changes: Simple stains with Pfeiffer's solution were made following the supersonic wave treatment (after one platinum loop portion of the test solution placed on the object plate had been dried and fixed). Microscopic observations on the destruction of bacterial cells were performed.

G. Measurement of turbidity: The bacterial suspensions were diluted with a physiological saline solution, according to the ratios shown below, and were measured with Pulfrich's photometer. Upon obtaining the relative turbidities the absolute turbidities were computed.

Bacterial solution concentration	Dilution
0.1 mg/10 cc	Stock solution
1.0 mg/1.0 cc	10 times
10.0 mg/1.0 cc	100 times

Chapter IV. Results of experiment

(See Tables 1, 2 and 3.)

Table 1. Results of cellulicidal strength tests; turbidity and survival of cholera bacteria treated with 1120 kc supersonic waves.

Plate voltage 3000 v; plate current 160 ma; grid current 165 ma. Date of experiment 21 Apr 41. Room temperature 22°C. Weather cloudy.

0.1 mg							1.0 mg							10.0 mg						
Time (min)	Survival of bacteria		Turbidity	Bacterial count - 0.1 mg (Z)	Survival compared to control (%)	Log Z	Time (min)	Survival of bacteria		Turbidity	Bacterial count - 1.0 mg (Z)	Survival compared to control (%)	Log Z	Time (min)	Survival of Bacteria		Turbidity	Bacterial count - 10.0 mg (Z)	Survival compared to control (%)	Log Z
	Peptone	Agar slant						Peptone	Agar slant						Peptone	Agar slant				
K	+	+	0.0166	59.81×10^6			K	+++	+++	0.[illegible]7	58810×10^6		8.7694	K	++	++	0.6246	5881×10^6		[illegible].7695
1	+	+	0.0133				1	+++	+++	0.[illegible]02				1	++	++	0.6023			
2	+	+	0.0120				2	+++	+++	0.[illegible]82				2	++	++	0.5688			
3	+	+	0.0117	0.06×10^6	0.102	5.7781	3	++	+++	0.[illegible]26				3	++	++	0.5464			
4	—	—	0.0120				4	+++	+++	0.[illegible]49				4	++	++	0.5333			
5	—	—	0.0150				5	++	++	0.[illegible]71	54.29×10^6	9.237	7.7334	5	++	++	0.5130			
6	—	—	0.0154				6	++	++	0.[illegible]03				6	++	++	0.4796			
7	—	—	0.0148				7	+	+	0.[illegible]71				7	++	++	0.4796			
8	—	—	0.0149				8	+	+	0.[illegible]47	9.16×10^6	1.558	6.9582	8	++	++	0.5130			
9	—	—	0.0146				9	+	+	0.[illegible]16				9	++	++	0.5353			
10	—	—	0.0136				10	+	+	0.[illegible]24	67	0.000011	1.9261	10	++	++	0.5800			
							15	—	+	0.[illegible]48				20	+	+	0.59116	5.43×10^6	0.092	6.7340
							20							40	+	+	0.75847	3.37×10^6	0.057	6.5302
														50	+	+	0.70333	0.6826×10^6	0.00147	4.9251
														60	—	—	0.61347			

Table 2. Results of cellulicidal strength tests; turbidity and survival of cholera bacteria treated with 560 kc supersonic waves.

Plate voltage 3600 v; plate current 530 ma; grid current 130 ma. Date of experiment 31 May 41. Room temperature 26°C.. Weather clear.

0.1 mg							1.0 mg							10.0 mg						
Time (min)	Survival of bacteria		Turbidity	Bacterial count - 0.1 mg (Z)	Survival compared to control (%)	Log Z	Time (min)	Survival of bacteria		Turbidity	Bacterial count - 1.0 mg (Z)	Survival compared to control (%)	Log Z	Time (min)	Survival of bacteria		Turbidity	Bacterial count - 10.0 mg (Z)	Survival compared to control (%)	Log Z
	Peptone	Agar slant						Peptone	Agar slant						Peptone	Agar slant				
K	++	+	0.0032	47.858×10^6		78.4515	K	+++	+++	0.1[illegible]039	678.585×10^6		8.8315	K	+++	+++	0.62462	705.85×10^6		9.8313
1	++	+	0.0071A				1	+++	+++	0.07A7[illegible]				1	+++	+++	0.52886			
2	—	—	0.00[illegible]8				2	+++	+++	0.0641[illegible]				2	+++	+++	0.45078			
3	—	—	0.0070[illegible]				3	+++	+++	0.0507[illegible]				3	+++	+++	0.45731			
4	—	—	0.00634				4	+++	+++	0.0483[illegible]				4	+++	+++	0.45174			
5	—	—	0.00436				5	+++	+++	0.05130	6.271×10^6	0.0396	5.4230	5	+++	+++	0.44058			
6	—	—	0.00477				6	+++	+++	0.0[illegible]66				6	+++	+++	0.43501			
7	—	—	0.00692				7	+++	+++	0.0479[illegible]				7	+++	+++	0.40154			
8	—	—	0.00747				8	+++	+++	0.0546[illegible]				8	+++	+++	0.40154			
9	—	—	0.00824				9	+++	+++	0.0580[illegible]				9	++	+++	0.52826			
10	—	—	0.00781				10	++	++	0.0602[illegible]				10	++	+++	0.40154			
15	—	—	0.00868				15	++	++	0.0638[illegible]	0.014×10^6	0.0036	4.1621	20	++	+++	0.42369	0.348×10^6	5.3345	
20	—	—	0.00903				30	+	+	0.0577[illegible]	459	0.0006	2.6532	30	++	+++	0.34577			
							40	—	—	0.0602[illegible]				40	++	+++	0.37934	0.023×10^6	0.0036	4.3627
							60	—	—	0.0586[illegible]				50	+	++	0.37934	0.007×10^6	0.0013	3.8451
														60	+	+	0.45753	0.0022×10^6	0.0003	3.3010
														75	—	—	0.35693			
														90	—	—	0.28000			

Table 3. Results of cellulicidal strength tests; turbidity and survival of cholera bacteria treated with 2MV kc supersonic waves.

Plate voltage 3300 v; plate current 400 ma; grid current 190 ma. Date of experiment 7 Jul 41. Room temperature 21°C. Weather clear.

0.1 mg						1.0 mg						10.0 mg					
Time (min)	Survival of bacteria		Turbidity	Bacterial count - 0.1 mg (Z)	Survival compared to control (%)	Log Z	Time (min)	Survival of bacteria		Turbidity	Bacterial count - 1.0 mg (Z)	Survival compared to control (%)	Log Z	Time (min)	Survival of bacteria		Turbidity	Bacterial count - 10.0 mg (Z)	Survival compared to control (%)	Log Z
	Peptone	Agar slant						Peptone	Agar slant						Peptone	Agar slant				
K	+	+	0.10948	58.430×10^6		7.7614	K	+	+	0.07361	4.200×10^6		8.7664	K	+	+	0.89539	58.1×10^6		9.7651
1	+	+	0.01374	1.522×10^6	7.783	6.6551	1	+	+	0.06558				1	+	+	0.90136			
2	—	—	0.01326				2	+	+	0.06525				2	+	+	0.58238			
3	—	—	0.01953				3	+	+	0.06134	4.768×10^6	4.280	8.3927	3	+	+	0.55780			
4	—	—	0.01367				4	+	+	0.06023				4	+	+	0.49685			
5	—	—	0.01339				5	+	+	0.06860	3.833×10^6	2.710	7.1995	5	+	+	0.49077			
6	—	—	0.01939				6	—	—	0.06470				6	+	+	0.45731			
7	—	—	0.01025				7	—	—	0.06492				7	+	+	0.41370			
8	—	—	0.00960				8	—	—	0.07361				8	+	+	0.42585			
9	—	—	0.00942				9	—	—	0.06860				9	+	+	0.41827			
10	—	—	0.00960				10	—	—	0.07138				10	+	+	0.41270	730.719×10^6	3.995	8.3617
							15	—	—	0.06681				15	+	+	0.08960	53.740×10^6	0.930	8.7213
														20	+	+	0.39397	0.246×10^4	0.004	5.7303
														30	—	—	0.39089			
														40	—	—	0.36808			
														50	—	—	0.33976			

The cellulicidal time is shown in Figure 1, the morphological changes in Table 4 and turbidities in Tables 1, 2 and 3.

A. Cellulicidal time

(Summarized from Tables 1, 2 and 3.)

		0.1 mg/1 cc	1.0 mg/1 cc	10.0 mg/1 cc
1	1120 kc	4 min	15 min	60 min
2	560 kc	2 min	30 min	75 min
3	280 kc	2 min	6 min	30 min

At the same supersonic wave energy, cellulicide was accelerated as the bacterial dilution was increased and as the frequency was decreased. At 280 kc cellulicide occurred in the 0.1 mg solution in two minutes, the 1.0 mg solution in six minutes and the 10.0 mg solution in 30 minutes. Higher destruction occurred at 1120 kc than at 560 kc. Consequently, the cellulicidal time (directly proportionate to wave length; inversely proportionate to frequency) fluctuated according to differences in wave length.

B. Survival test: As illustrated in Figures 2 and 3 the decrease in bacterial count produced by treatment with supersonic waves is logarithmical. By treating the bacterial count in the graphs as a logarithmic scale (vertical axis) and the treatment time as an arithmetical scale (horizontal axis) it can be said that the relationship

$$\log Y = cx \qquad Y = Ke^{cx}$$

(c = negative constant)

exists (when Y represents the bacterial count and X represents the treatment time) since approximately straight lines are shown when viewed in terms of logarithmic values for the number of surviving bacteria.

In the case of the 10.0 mg/1.0 cc solution graphed in Figure 3 a marked reduction in bacterial count occurs at the same energy level when the frequency decreases and the wave length increases. This reduction is not pronounced in Figure 2.

The time required for the logarithm of the live bacteria count in the suspensions of each concentration to become halved, or their fractional survival time, is shown below.

		0.1 mg	1.0 mg	10.0 mg
1	1120 kc	3 min	5 min	40 min
2	560 kc	1 min	15 min	30 min
3	280 kc	1 min	5 min	20 min

C. Results on morphological changes: (See Table 4.)

The influences exerted by frequencies on the bacterial cells, when expressed in terms of time required for cellulicide, have been summarised in the following table.

Frequency (kc)		1120			560			280		
Bacterial weight (mg)		0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0
Items examined	(1) Normal endurance period of cells	(min) 3	(min) 9	(min) 50	(min) 1	(min) 20	(min) 50	(min) 1	(min) 5	(min) 20
	(2) Inability to stain	-	-	4	-	2	2	-	2	5
	(3) Cellular swelling	-	3	4	-	1	1	1	1	1
	(4) Cellular degeneration	-	3	6	-	-	-	-	2	10
	(5) Protoplasm (homogenization)	-	4	8	-	3	3	-	1	10
	(6) Granular precipitate	1	3	5	9	4	1	-	-	9
	Cloudy precipitate	1	2	8	15	8	3	9	-	9
	Dust-like precipitate	1	2	6	7	1	1	1	1	2

The steps taken in the destruction of bacterial cells by means of vibrations included cellular swelling followed either by changes in the cell morphology or by immediate destruction and homogenization upon the release of protoplasm. The destroyed matter from the bacterial cells displayed dust-like, granular or cloudy forms. As a peculiarity of supersonic wave treatments certain parts were unable to take stains. The above "steps" were taken in rapid succession at 280 kc.

D. Results on measurement of turbidity: (See Tables 1 to 3.)

Within the range of supersonic wave treatments covered by this experiment, transparency was not detected among any of the suspensions of cholera bacteria. In every case extreme differences in turbidity were not present.

Chapter V. Summary and conclusion.

The following conclusions were derived from observing the cellulicidal time as well as changes in the turbidity and the morphology of bacterial solutions after subjecting cholera bacteria suspended in a physiological saline solution to the action of supersonic waves possessing the same energy level but of a different frequency (1120 kc, 560 kc and 280 kc).

A. At the same energy level the cellulicidal time is accelerated as the frequency is decreased and is inversely proportionate to the frequency (somewhat directly proportionate to wave length).

B. Cellulicidal time decreases as the concentration of the bacterial suspension decreases.

C. The changes occurring in live bacteria during the treatment time are logarithmical and possess the following relationship:

$$\log y = cx$$

y = live bacteria count

x = treatment time

c = negative constant

D. Hardly any case of transparency due to the effects of supersonic waves on cholera bacteria and physiological saline solution can be observed. Turbidity appears unchanged.

E. The steps taken in cellular destruction by supersonic wave treatment include cellular swelling and deformation or immediate destruction and homogenization upon the release of protoplasm. The destroyed matters display dust-like, granular or cloudy forms.

F. The degree of cellular destruction cannot be learned through changes in turbidity.

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Table 4. Microscopic observations of morphological changes in cholera bacteria at various supersonic wave frequencies.

Frequency			1120 kc									560 kc									280 kc								
Microscopic observations			Cellular swelling	Morphological deformation	Cellular destruction	Protoplasm (homogenization)	Granular	Cloudy	Dust-like	Normal	Inability to stain	Cellular swelling	Morphological deformation	Cellular destruction	Protoplasm (homogenization)	Granular	Cloudy	Dust-like	Normal	Inability to stain	Cellular swelling	Morphological deformation	Cellular destruction	Protoplasm (homogenization)	Granular	Cloudy	Dust-like	Normal	Inability to stain
Bacterial solution concentration		Time																											
	0.1 mg	K	—	—	—	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		1	—	—	+	—	+	—	+	++	—	—	—	+	—	—	—	—	++	—	+	—	+	—	—	—	—	++	—
		2	—	—	++	—	+	—	+	+	—	—	—	++	—	—	—	—	+	—	—	—	++	—	—	—	—	+	—
		3	—	—	++	—	++	—	+	+	—	—	—	++	—	—	—	—	+	—	—	—	++	—	—	—	—	+	—
		4	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	—	+	—	—	—	++	—	—	—	—	+	—
		5	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	+	+	—	—	—	++	—	—	—	—	+	—
		6	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	+	+	—	—	—	++	—	—	—	—	+	—
		7	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	+	+	—	—	—	++	—	—	—	—	+	—
		8	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	+	+	—	—	—	++	—	—	—	—	+	—
		9	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	+	+	—	—	—	++	—	—	—	—	+	—
		10	—	—	++	—	++	—	+	—	—	—	—	++	—	—	—	+	+	—	—	—	++	—	—	—	—	+	—
		15										—	—	++	—	+	++	+	—	—									
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	1.0 mg	K	—	—	—	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		1	—	—	—	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		2	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		3	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		4	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
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		10	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		15	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		20	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		30	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		40	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
	10.0 mg	K	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		1	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		2	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		3	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		4	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		5	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		6	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		7	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		8	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		9	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		10	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		15	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		20	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		25	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		30	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		40	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		50	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		60	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		75	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—
		120	+	+	+	—	—	—	—	++	—	—	—	—	—	—	—	—	++	—	+	—	—	—	—	—	—	++	—